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Deletion of the mental retardation gene *Gdi1* impairs associative memory and alters social behavior in mice

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Non-specific mental retardation (NSMR) is a common human disorder characterized by mental handicap as the only clinical symptom. Among the recently identified MR genes is *GDI1*, which encodes αGdi, one of the proteins controlling the activity of the small GTPases of the Rab family in vesicle fusion and intracellular trafficking. We report the cognitive and behavioral characterization of mice carrying a deletion of *Gdi1*. The *Gdi1*-deficient mice are fertile and anatomically normal. They appear normal also in many tasks to assess spatial and episodic memory and emotional behavior. *Gdi1*-deficient mice are impaired in tasks requiring formation of short-term temporal associations, suggesting a defect in short-term memory. In addition, they show lowered aggression and altered social behavior. In mice, as in humans, lack of *Gdi1* spares most central nervous system functions and preferentially impairs only a few forebrain functions required to form temporal associations. The general similarity to human mental retardation is striking, and suggests that the *Gdi1* mutants may provide insights into the human defect and into the molecular mechanisms important for development of cognitive functions.

INTRODUCTION

Mental retardation (MR) is a common human disorder that may result from genetic causes, environmental insults or a combination of the two. MR may be one of the clinical signs of a syndrome, as in Down syndrome, or it may be associated with metabolic, mitochondrial or developmental disorders. A large group of MR includes the non-specific forms (NSMR), whose only consistent clinical manifestation is mental handicap. As NSMR is most likely caused by alterations in molecular pathways important for cognitive functions, defining the relevant genes might help in defining brain functions for intellectual and learning abilities.

Insights into the NSMR group have come from the study of X-linked MR (XLMR), which represents ~5% of all MR, corresponding to a prevalence in the general population of 1 in 600 males. The study of large X-linked families has helped to distinguish different forms of NSMR through regional assignment along the X chromosome, and over the last few years has contributed to the identification of several XLMR genes (1–4).

One XLMR gene is *GDI1* (5), which encodes one of the proteins regulating the small GTPases of the Rab family, a group of small Ras-like GTPases, involved in vesicle fusion in the exocytic and endocytic pathways (6). The Rab GTPases cycle between an active GTP-bound and an inactive GDP-bound state through the action of regulatory proteins. Among the regulatory proteins, GDIs are required to retrieve the GDP-bound form from the membrane and to maintain a pool of soluble Rab-GDP (7,8). Two different GDIs have been described in mammals (9–11). They interact with >40 Rab proteins and participate in fusion of different cellular membranes.

Since αGDI encoded by *GDI1* is the most abundant form in brain (10), it was suggested that the main consequence of mutations in *GDI1* could be a modification of the pool of Rab3A, the most abundant of the Rab proteins in brain (12,13), leading eventually to alterations of synaptic vesicle exocytosis, similar to those described in the mouse knockout (KO) for *Rab3A* (14–16). *GDI1*, however, may also be relevant through

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its general role in the endo/exocytic pathways. The finding that in the mouse, *Gdi1* is expressed early in development and is upregulated at early stages of brain differentiation, suggested that it may also play a role in neuron migration and/or differentiation (5). Accordingly, we have shown that rat hippocampal neurons and PC12 cells treated with *Gdi1* antisense oligonucleotides showed inhibition of axonal outgrowth (5,17). Recently, *Gdi1* constitutive KO mice were produced (18): a thorough electrophysiological analysis showed that the mutant mice presented alterations in short-term synaptic plasticity. For many electrophysiological parameters, the absence of *Gdi1* had quite different consequences compared with that of Rab3A (14,15), and in general the electrophysiology results were inconsistent with the hypothesis that lack of *Gdi1* acts by disrupting Rab3A function.

In this study, we present the cognitive and behavioral characterization of *Gdi1*-deficient mice generated in our laboratory, and we suggest that they represent a good model to study the role of human *GDI1* in the establishment of cognitive functions and in MR. MR is a very heterogeneous disorder, and the identification of a small number of genes responsible for the disorder has demonstrated that the number of patients carrying mutations in any of the genes is very low (1,4). Thus the study of the molecular mechanisms causing human MR requires the use of animal models. Despite the obvious difference in cognitive functions between mouse and human, it is not unlikely that basic molecular mechanisms common to both species may be altered by mutations in MR genes and that their identification in the mouse will lead to new insights into the human defects. This seems to be the case for the *Gdi1* KO mice, which are normal from the anatomical point of view, as described for patients affected by NSMR. Similarly, mentally retarded people are defined as patients presenting impaired cognitive functions, causing a lowered IQ, as well as altered behavior (49). This was strikingly found also in the *Gdi1* KO mice that presented specific alterations in forming and retaining temporal associations as well as altered social behavior.

RESULTS

Generation of mice carrying a null *Gdi1* gene

To generate *Gdi1*-null mice, we constructed a targeting vector in which exons 1–4 of the mouse *Gdi1* gene were substituted by the *LacZ* gene under the *Gdi1* promoter and a neomycin-resistance gene cassette transcribed from the *PGK1* promoter (Fig. 1A). Two homologous recombinant clones having the highest percentage of normal caryotypes (93%) were injected into blastocysts. Chimeras were obtained from both ES cell clones and were crossed to C57BL/6J and B6D2F1 (C57BL/6J × DBA2) females.

The heterozygote females were crossed to WT males of the corresponding genetic background. Wild-type (WT) and KO males from the crosses (F2) were analyzed, as well as WT and KO mice born from F2 heterozygote females crossed to WT males of the same generation and of the same genetic background (F3). Backcrosses into the C57BL/6J genetic background were done, and the fifth generation (N5) was used in many tests. For all animals, the genotype was determined by PCR, using the primers G1/G2 and Z1/Z2 (Fig. 1B) and by Southern blot (Fig. 1C). RT–PCR analysis of RNA extracted from KO mice showed that the *Gdi1* mRNA was not produced (not shown). Western blot analysis of total protein extracts from brain confirmed the result, showing that αGdi was absent from brains of KO mice (Fig. 1D).

The *Gdi1*-null mice were viable and fertile: the mutant allele was transmitted in the expected Mendelian segregation ratio of an X-chromosome gene. Both male and female KOs appeared normal and healthy, indicating that also in the mouse the *Gdi1* gene is not essential for life. No differences were seen between generations and genetic backgrounds, in all the experiments described: the same results were obtained in two mixed backgrounds tested (129B6 and 129B6D2) and in the fifth-generation backcross of the KO into the C57BL/6J, where the 129 genetic contribution is reduced to $<10\%$. Moreover, the results were identical in several different litters of all the generations studied, carrying presumably different portions of the 129 and C57Bl/6J and DBA2 genomes.

Histological analysis

Microscopic examination of the brains of age-matched adult WT and KO animals of the F2 and F3 generations did not show major differences. *Gdi1* appeared to be ubiquitously expressed, as determined by *in situ* hybridization of transverse and coronal sections of WT animals (Fig. 2). The highest expression was detected in the hippocampus, in the piriform cortex, in nuclei of the hypothalamus and of the amygdala, in part of the layer V of cortex, in the olfactory bulb, and in the Purkinje cells of the cerebellum. Histological staining and comparison between WT and KO mice demonstrated that the organization of most regions of the brain was unaltered by the KO. The only area in which we could detect alterations was the hilar part of the CA3 region of the hippocampus (Fig. 3). By Nissl stain, the CA3 pyramidal cells in the KO appeared more scattered than in WT. Comparison of cell counts (in five WT and five KO animals) did not reveal differences in cell density, and suggested developmental anomalies in layering of pyramidal neurons. By Timm staining, a trilamination of suprapyramidal mossy fibers was observed in consecutive horizontal sections of both hemispheres in 77% of the sections of nine 129B6 and in 65% of three 129B6D2 KO animals analyzed. Trilamination of suprapyramidal mossy fibers was detected also in WT animals, but at much lower frequency: in 33% and 11% of the sections of eleven 129B6 and four 129B6D2 WT littermates, respectively.

Levels and subcellular distribution of Rab GTPases and other proteins

We determined the level of Rab proteins and of a wide range of proteins involved in fusion and recycling of synaptic vesicles in total extracts of brains of WT and KO animals of the F2 and F3 generations of both genotypes. Equal amount of proteins were fractionated by acrylamide gels and after western blot the amount of protein in each lane of the gel was controlled by reaction with anti-MAPK antibodies. After reaction with specific antibodies, the amount of protein in each band was

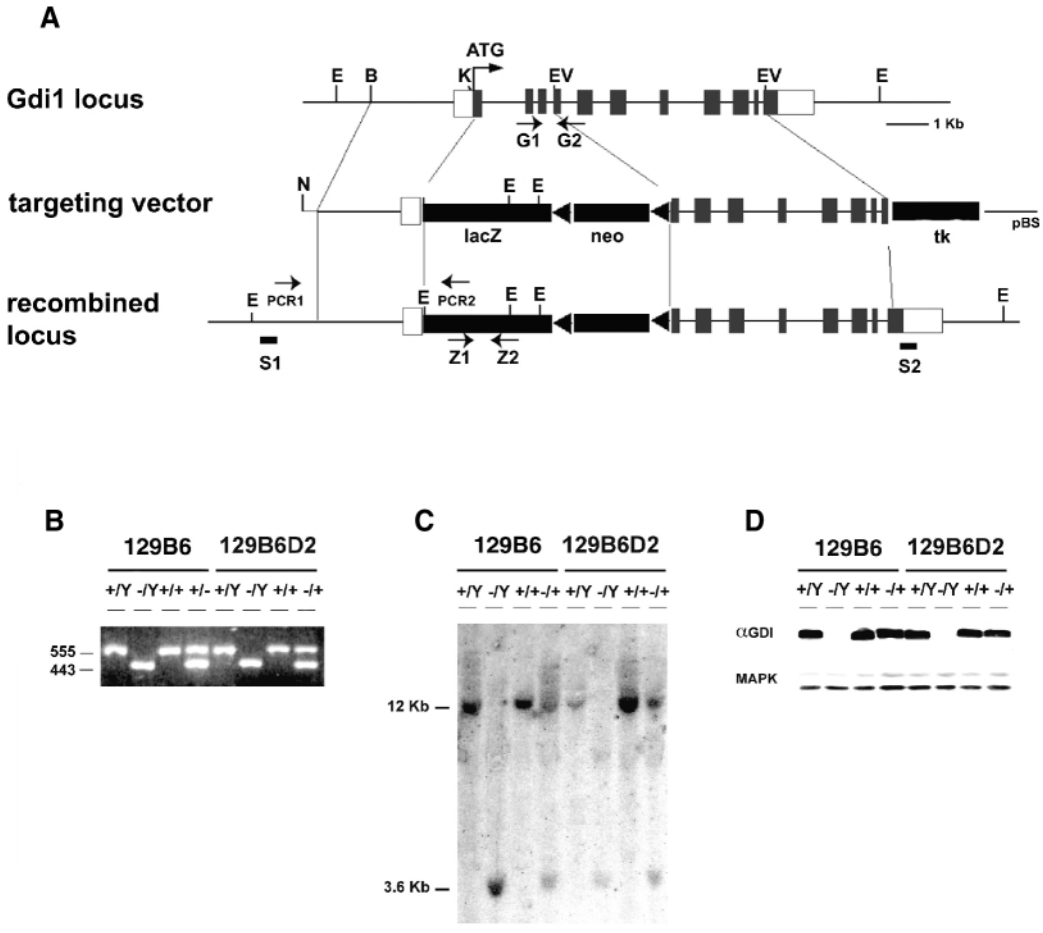

Figure 1. Targeted disruption of the *Gdi1* gene by homologous recombination. (**A**) Scheme of the structural organization of the *Gdi1* gene (top), of the targeting vector (middle) and of the mutated *Gdi1* gene (bottom). Black boxes are coding exons or the indicated insertion cassettes; white boxes are non-coding regions. Restriction enzymes are *Eco*RI (E), *Bam*HI (B), *Kpn*I (K), *Eco*RV (EV) and *Not*I (N). The position of the PCR primers for the screenings (PCR1/PCR2; G1/G2; Z1/Z2) is indicated by arrows. S1 and S2 are the probes for Southern blot analysis. (**B**) PCR analysis of DNA extracted from the tails of F2 mice, using the primers G1/G2 and Z1/Z2. (**C**) Southern blot analysis of F2 mice. Tail DNAs were digested with *Eco*RI, fractionated on agarose gel and hybridized with the 5′ flanking probe S1. The 12 kb *Eco*RI fragment corresponds to the WT locus, and the 3.6 kb fragment to the recombinant locus. (**D**) Western blot analysis of αGdi in total brain proteins from the F2 mice indicated. MAPK was used as a quantitative control marker.

measured by densitometry and the ratio between the WT and KO bands was calculated. None of the synaptic proteins studied, irrespective of their localization in synaptic vesicles (SV2, Stg-1,2, p38/Syp, Rbph-3A, Synapsins and VAMP-2), presynaptic membranes (NSF, Munc-18, Stx-1, α,β-SNAP, SNAP-23 and SNAP-25) or involved in endocytosis (dynamin, synaptojanin and amphiphysin), presented an altered level (not shown).

The situation was quite different for the Rab GTPases (Fig. 4A). They could be grouped in three groups. Rab3A was present in similar levels in WT and KO animals (KO/WT 0.98). A small decrease was seen in the KO brains compared with WT in the amounts of Rab3D, Rab3B, Rab1, Rab7 and Rab8 (KO/WT 0.70–0.79). A greater difference was observed in Rab4, Rab5, Rab3C and Rab11 (KO/WT 0.40–0.53). Similar results were obtained for all genotypes and generations studied.

To determine whether the reductions in the steady-state level of some of the Rab proteins were due to decreased synthesis, semiquantitative RT–PCR was done from total RNA from brains of WT and KO littermates of the F3 generation, in the two genetic backgrounds. The two different transcripts of Rab5 and Rab11 were analyzed. All Rab RNAs were present in identical levels in mutants and WT mice (not shown).

We also determined whether an increase in the concentration of βGDI in brain of the KO mice could explain the relatively small effect of the absence of αGDI on some of the Rab proteins. In the total brain extract preparations, βGDI concentration was unchanged (Fig. 4A).

The Rab GTPases were analyzed by western blot in subcellular fractions from cortex or from subcortical brain regions. (Fig. 4B) shows the results of the fractionation of the cortex of 129B6 mice. Subcellular fractionation was obtained by serial centrifugation at progressively higher speeds, as described (19). As a control of the quality of the fractionation, all gels were reacted with anti-synaptotagmin antibodies; anti-MAPK antibodies were used as control of the total proteins, in each subcellular fraction (Fig. 4B). This analysis confirmed the data obtained from the total extracts,

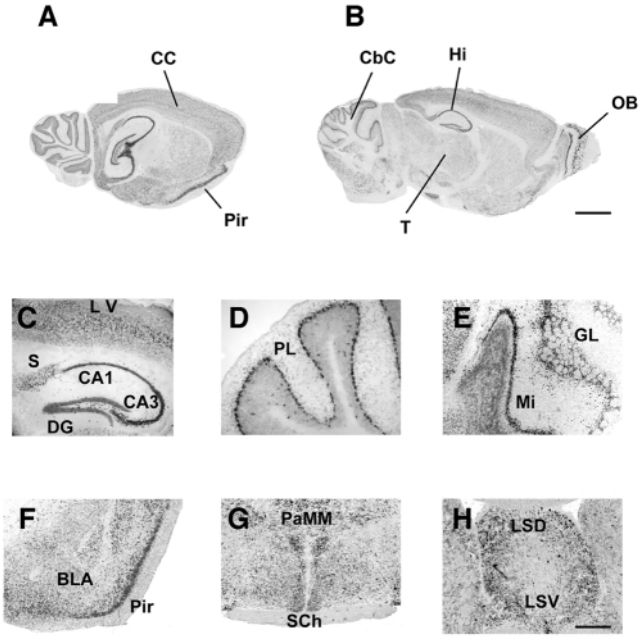

Figure 2. *In situ* hybridization analysis of *Gdi1* WT mice. Light-microscope images showing the distribution of *Gdi1* mRNA in brain sections of WT mice. (**A** and **B**) Two different sagittal sections of a 4-month-old WT mouse. (**C**)–(**H**) Enlargements of specific regions of saggital (C, D and E) and coronal sections (F, G and H). CC, cerebral cortex; Pir, piriform cortex; CbC, cerebellar cortex; Hi, hippocampus; OB, olfactory bulb; T, thalamus; L V, layer V of cerebral cortex; S, subiculum; CA1 and CA3, fields of hippocampus; DG, dentate gyrus; PL, Purkinje layer; GL, glomerular layer; Mi, mitral cells; BLA, basolateral amygdala; PaMM, paraventricular hypotalamic area, medial, magnocellular part; SCh, suprachiasmatic nucleus; LSD, lateral septal nucleus, dorsal part; LSV, lateral septal nucleus, ventral part. Scale bars: 2 mm (A and B) and 0.1 mm (C–H).

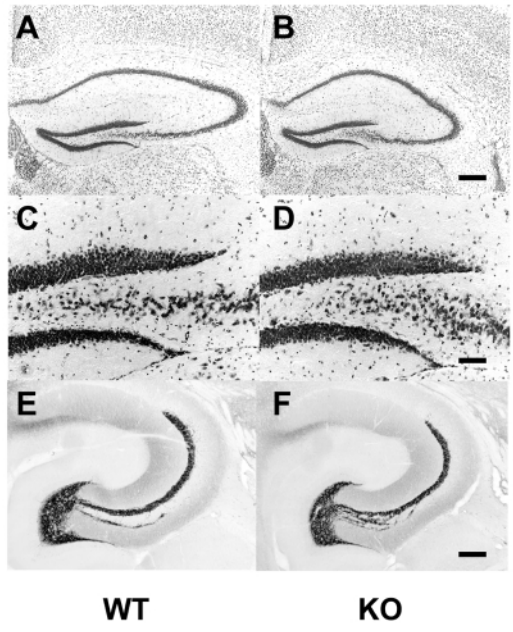

Figure 3. Lamination defect in the CA3 region of the hippocampus. (**A** and **B**) Nissl staining of coronal sections in the area of the hippocampal formation (magnification ×5). (**C** and **D**) Greater magnification (×20) of the same sections to show the CA3 region. (**E** and **F**) Mossy fiber projections selectively stained with the Timm method on horizontal sections. In contrast to the two clearly separated laminae in WT, mutant mice were characterized by a disintegrated lower projection (magnification ×5). Scale bars: 0.5 mm (A, B, E and F) and 0.025 mm (C and D).

and showed that the ratio between membrane-bound and soluble Rabs in WT and in mutant mice was not identical for all the Rabs tested. The amount of Rab3A was only very slightly increased in the membrane fractions (LP1 and LP2) of the KO, and not very much decreased in the soluble fractions. Moreover, the amount of Rab3A in the synaptosomal fractions (LP2 and LS2), where most Rab3A is localized, was similar in WT and KO animals. Reduction of Rab3A was visible in the supernatant of the cytoplasmic fraction, where it is, however, present in low amount. Reduced levels of the soluble form were seen for all the other Rab proteins tested. The soluble form of the more abundant Rab proteins, Rab4 and particularly Rab5, was very much reduced in all fractions (S3, LS1 and LS2), and almost absent in the synaptosomal fractions (LS2). No differences were detected between extracts from the different brain regions and the two genetic backgrounds studied.

Exploration, fear-related behavior and motor abilities are normal in *Gdi1*-mutant mice

We assessed exploratory and emotional behavior of the mice in the open field (20), in an elevated circular maze (O-maze) (21) and in a dark/light box (22). We also checked muscle strength and motor coordination in various tests, including the rotarod (23), footprint patterning (23), moving along a wire coat hanger (24), ridged-beam walking (24) and grip strength (25) (not shown). In all the tests, ≥ 13 animals were used for each genotype and background. ANOVA analysis did not reveal statistically significant differences ($P > 0.5$) between WT and mutant mice of both genetic backgrounds in any of the tests performed.

Gdi1-mutant mice do not show impairment in spatial learning in the Morris water maze

The Morris water maze task has been used extensively to study spatial learning in rats (26) and in mice (27). Mice were subjected to the standard hidden-platform version of the Morris water maze (28). The WT and *Gdi1* KO mice (from the F2 and F3 generations of both genetic backgrounds tested) were subjected to 3 days of six training trials per day with the platform in the same position (acquisition phase). On the fourth day, the platform was moved to a different position, and mice were subjected to 2 days of six trials per day (reversal phase). In neither sessions was there a significant difference between WT and KO mice in the time to reach the platform during the trials (Fig. 5A). Both genotypes showed significant decrease in escape latency during both phases [ANOVA by repeated exposures; $F(14,8) = 17.17$, $P < 0.0001$] and no significant difference between genotypes [ANOVA genotypes by repeated exposures; $F(1,80) = 2.87$, $P = 0.19$]. No differences were observed between WT and KO mice during the probe trial in crossing the former goal annulus and in swimming in a circular

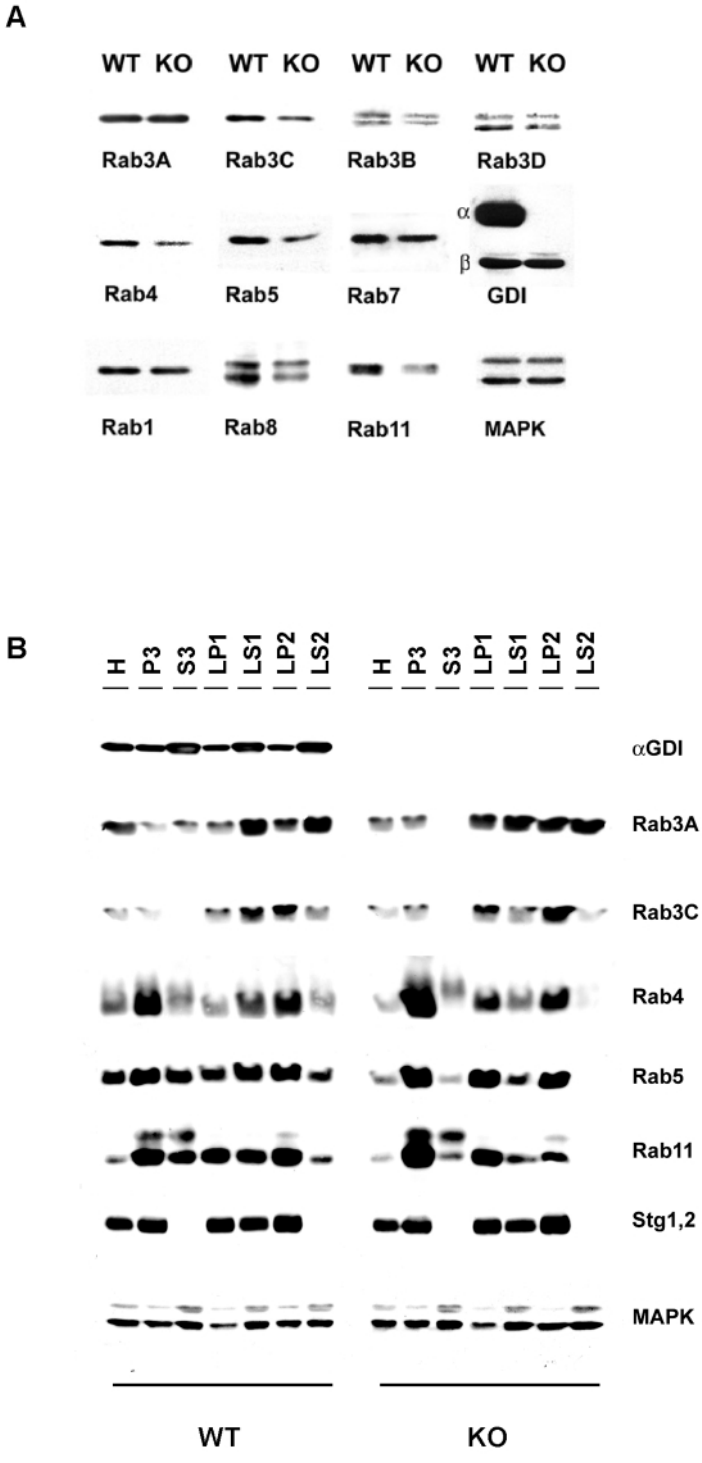

Figure 4. Analysis of βGDI and Rab proteins in cortex of WT and KO mice. (**A**) Western blot of total extracts of two pooled brain cortexes of 129B6D2 mice, fractionated on 10% SDS–PAGE gels and analyzed with the antibodies to the Rab proteins indicated below each band and with a commercial anti-GDI antibody (Zymed) that recognizes both α- and βGDI. MAPK was used as a control of the amount of proteins in the gel. (**B**) Western blots of pooled brain cortex fractions of six 129B6 mice. Antibodies to the Rab proteins are indicated. The anti-Gdi antibody used in the experiment was a monoclonal antibody (a generous gift from Dr R. Jahn) that did not interact with βGDI. The amounts of proteins were normalized using MAPK. The quality of the fractionation was controlled using antibodies to synaptotagmin (Stg1,2). Fractions are indicated above the lanes: H, total homogenate; P3, cell body membranes; S3, cytosol; LP1, mitochondria and pre- and postsynaptic membranes; LS1, total synaptosomal fraction; LP2, synaptic vesicle fraction; LS2, synaptosol.

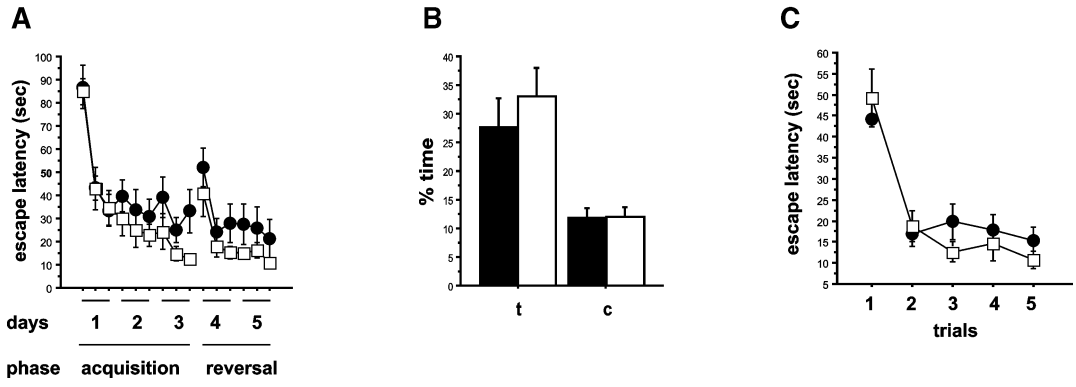

Figure 5. Spatial memory and episodic-like memory in the Morris water maze. (**A**) Pooled results obtained from 129B6D2 and 129B6 mice from the F2 and F3 generations (KO, $n = 40$; WT, $n = 43$) in the standard version of the vater maze. Learning curve indicating the time required to reach the hidden platform (escape latency expressed in seconds) over 5 days. (**B**) Probe trials (first 30 s of the first trial of reversal phase) expressed as the percentage of time spent in a circular zone around the trained goal (t) and the averaged control goals (c). Chance level of 12.5%. Data are from the same experiment as shown in (A). (**C**) DMP version of the Morris water maze using N5 mice (KO, $n = 13$; WT, $n = 13$). Latencies (in seconds) of the first five trials during new platform training, averaged from the last two training sessions (4 and 5 platform positions). Bars represent the standard error. Open squares are WT animals and closed circles are KO.

zone around it (Fig. 5B) [ANOVA genotypes by repeated exposures; $F(1,80) = 2.01$, $P = 0.15$].

Gdi1-mutant mice do not show impairment in episodic-like memory in the delayed matching-to-place task

N5 backcross mice were also tested in a modified water maze protocol—the delayed matching-to-place task (DMP) (29,30). In this version of the Morris water maze, mice have to learn different platform positions during several trials, in different training sessions. Mice are trained to escape onto the hidden platform at one location, until they reach the criterion of three consecutive trials with an escape latency of ≤ 15 s, or they complete a maximal number of trials. When the criterion or all trials are achieved, a new training session begins on the next day, with the platform moved to a new position. This procedure is repeated until the mice learn five platform locations. In such a procedure, mice are able to quickly acquire the platform position of each training session, on a single exposure, as shown by the reduction of the escape latency in the second trial compared with the first trial within a training session. This performance reflects the ability of the mice to selectively retrieve the most recent platform location, an episodic-like component of the task, against the interference of the long-term memories encoded by the other locations.

During the first training session, there was no difference between WT and KO mice in the time to reach the platform, as expected from the results obtained with the F2 and F3 generations in the standard version of the Morris water maze. In the following sessions, WT and KO mice were able to acquire and remember each new platform position, reducing their escape latencies, across the first five trials of all training sessions (Fig. 5C). The analysis of the first five trials of the last two training sessions (platform positions 4 and 5) showed learning in both groups [$F(4,24) = 25.7$, $P < 0.0001$], and there was no significant difference between genotypes [ANOVA genotype by repeated exposure; $F(1,24) = 0.226$, $P = 0.639$].

Gdi1-mutant mice are impaired in spatial working memory in the radial maze

In the eight-arm radial maze test, a food-deprived mouse can obtain a food morsel at the end of each of the eight arms radiating from a center platform at equidistant points (31). Since food is not replaced during a trial, revisiting an arm is not rewarded with food, and is scored as an error. A trial began by placing the mouse on the center platform of the fully baited maze. The mouse was then allowed to choose freely among the arms until all eight food morsels had been obtained or until a total of 15 minutes had passed. Behavior was scored for the number of arms entered until all eight arms had been entered at least once and for the number of successive correct arm visits until an arm was revisited for the first time (position of the first repetition).

The *Gdi1* KO and WT littermates of the F2 and F3 generations of both genetic backgrounds and of the N5 backcross were tested for 10 trials, with one trial per day. Similar results were obtained. Figure 6 shows the results for the N5 backcross generation. The number of total errors declined over the 10 days of training in both WT and KO mice. However, acquisition in KO mice was significantly slower than that in WT (Fig. 6A) [ANOVA; $F(1,26) = 10.90$, $P = 0.0028$]. Eventually, the mutants also learned to patrol the maze, as indicated by a significantly decreased number of errors at the end of the task [ANOVA repeated exposure, calculated for mutants only; $F(14,4) = 9.98$, $P < 0.0001$] and no difference between WT and KO (Fig. 6A) [ANOVA for 'day 9–10' $F(1,26) = 1.30$; $P = 0.26$]. WT and KO mice differed highly significantly in the position of the first repetition (Fig. 6B) [ANOVA genotype by repeated exposure; $F(1,26) = 0.015$, $P = 0.0015$]. Whereas WT eventually reached almost perfect performance (seven out of a maximum number of eight correct successive arm visits), KO mice barely came above chance level performance (5.5 correct successive arm visits after 10 days of training). In conclusion, the *Gdi1* KO mice seem not to have defects in procedural learning, but to have a specific and quite severe deficit in working memory.

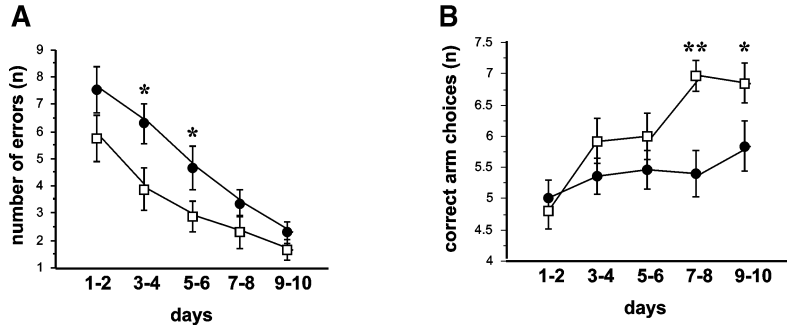

Figure 6. Radial maze task. KO ($n = 15$) and WT ($n = 13$) mice of the N5 backcross generation were tested for 10 days as described in Materials and Methods. (**A**) Mean number of errors until eight correct choices were made. (**B**) Learning performance expressed as the mean number of correct arm choices before the first error. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$. Data points represent the mean and bars the standard error. Open squares are WT animals and closed circles are KO.

Gdi1-mutant mice display deficits in trace but not delay fear conditioning

Auditory fear conditioning is a form of associative learning where an initially neutral tone is paired with the delivery of a brief foot shock as the unconditioned stimulus (US). The US by itself elicits jumping followed by freezing as the unconditioned reaction. Through conditioning, the tone becomes a conditioned stimulus (CS), which is able to elicit a conditioned reaction, such as freezing, resembling the unconditioned reaction. In the standard procedure—delay conditioning—the US is superimposed for a short period of time with the presentation of the CS (32). In trace conditioning, US presentation follows the end of CS presentation after a short interval has passed. The two forms of conditioning are dependent on different mechanisms: trace, but not delay, conditioning was shown to depend on an intact hippocampus (33,34).

Mice of both genetic backgrounds (17 KO and 17 WT) and the N5 backcross (7 KO and 7 WT) were analyzed with the delay conditioning protocol. No significant differences between WT and *Gdi1* KO mice could be found. Repeated presentation of the CS increasingly elicited freezing in both groups (Fig. 7A) [ANOVA for 'trial'; $F(1,48) = 38.66$, $P < 0.001$]. Baseline freezing scores during the two test sessions were close to zero; but freezing increased substantially 24 hours as well as 2 weeks later when the CS was presented during the second half of the test session (Fig. 7B and C) [$F(3,12) = 20.14$, $P < 0.001$ and $F(3,12) = 21.26$, $P < 0.001$, respectively].

In contrast, in trace fear conditioning, *Gdi1* KO froze significantly less than WT in the presence of the CS during conditioning as well as in the subsequent two test sessions, 3 days and 2 weeks later. Figure 7D–F shows the results of the N5 generation (16 KO and 16 WT). Identical results were obtained with the F2 and F3 generations of the two genetic backgrounds studied (29 KO and 28 WT tested). CS presentation elicited freezing in mutants that increased over the five conditioning trials; but this increase was significantly smaller than that of WT [trial × genotype; $F(1,30) = 15.06$, $P = 0.0005$]. Neither group froze in response to the test chamber alone during the two test sessions (Fig. 7E and F). Presenting the CS, however, during the second half of each session extensively and selectively increased freezing in WT; the response in mutants was only moderate or absent [3 days: $F(1,14) = 4.66$, $P = 0.04$; 2 weeks: $F(1,14) = 13.6$, $P = 0.002$].

The differences between *Gdi1* KO and WT mice cannot be explained by differences in pain sensitivity, since a pain threshold test did not reveal any group difference (not shown). Pain sensitivity was assessed by introducing the mice individually into the conditioning chamber and exposing them to increasing levels of shock intensity until a clear response could be observed (35).

Social interactions

Anecdotal observations of the mice handled in the animal house suggested that the *Gdi1* KO mice were less aggressive than their normal littermates. We assessed the aggressive behavior of the mice in the resident-intruder test (36). Both genetic backgrounds were tested using intruders of the corresponding genetic background. The test lasted 5 minutes for the 129B6D2 mice, which were more aggressive, probably because of their DBA2 component (37). The 129B6 mice were tested for 10 minutes. The results were similar with both backgrounds. Figure 8A, B and C shown results for the pooled F2 and F3 generations of the 129B6 mice. Aggressive behavior was greatly reduced in the mutants. Both the total aggression time and the number of attacks were significantly reduced for the KO mice. ANOVA revealed a significant difference between genotypes in the total aggression time, [$F(1,37) = 20.13$, $P < 0.0001$] and in the number of attacks [$F(1,37) = 9.47$, $P = 0.003$]. Only 10% of the KO mice attacked the intruder, compared with close to 75% for the WT. The rest of the animals remained neutral (30%) or were eventually attacked by the intruder (60%).

Also, the behavior of the KO mice with respect to the intruder was quite different from that of the WT mice. As soon as the intruder was introduced into the cage, the resident WT mouse, before attacking, sniffed the back and seldom the head of the intruder. In contrast, mutant resident mice spent the same time as the WT mice sniffing the back (data not shown), but a longer time sniffing the snout area of the intruder, as shown in Figure 8C. ANOVA revealed non-significant difference between genotypes in the time spent sniffing the back [$F(1,37) = 0.66$, $P = 0.41$], but a significant difference in the time spent sniffing the snout area [$F(1,37) = 4.83$, $P = 0.03$].

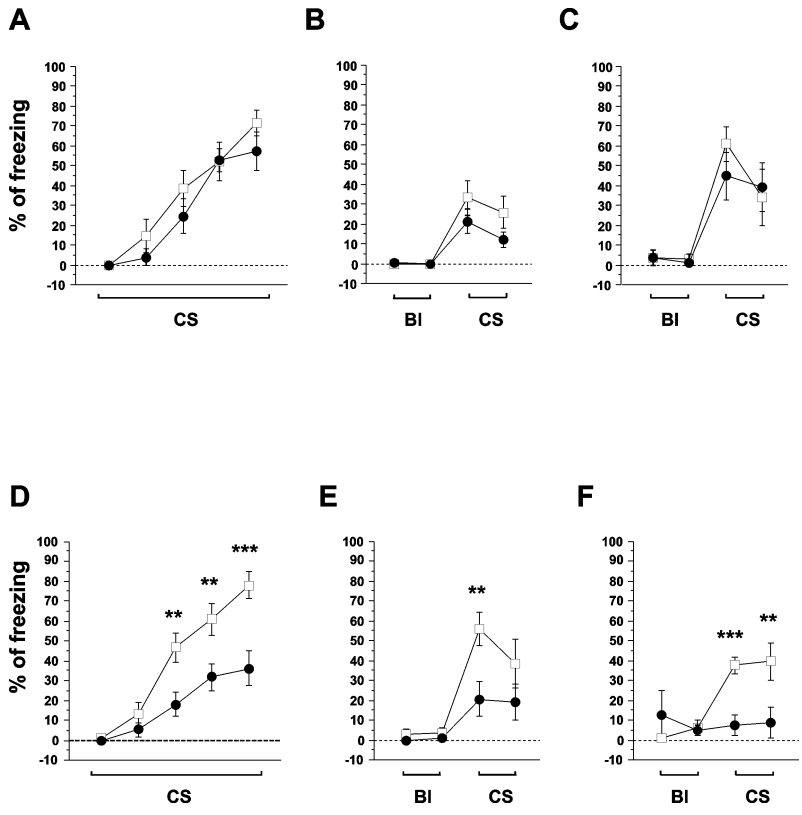

Figure 7. Fear conditioning task. (**A**, **B** and **C**) KO ($n = 7$) and WT ($n = 7$) N5 backcross animals tested for delayed fear conditioning: (A) average percentage of freezing during each 15 s CS presentation, during the training session; (B and C) average percentages of freezing during the memory test, 24 h (B) and 2 weeks (C) after (**D**, **E** and **F**) KO ($n = 16$) and WT ($n = 16$) N5 backcross animals tested for acquisition of trace fear conditioning: (D) average percentage of freezing displayed during each 15 s CS presentation, during the training session; (E and F) average percentages of freezing during the memory test 3 days (E: KO, $n = 8$; WT, $n = 8$) and 2 weeks (F: KO, $n = 8$; WT, $n = 8$) after training. The test was done as described in Materials and Methods. Data points represent mean freezing and bars the standard error, during the baseline (Bl, 30 s each) and during CS (15 s in A and D; 30 s in B, C, E and F). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. Open squares are WT animals and closed circles are KO.

The social behavior of the *Gdi1* KO mice is not due to olfactory problems or to altered testosterone levels

Social behavior in mice is strongly dependent on olfaction (38,39). Male-to-male and male-to-female recognition are also dependent on steroid hormone levels (40).

Olfaction was tested in two different and independent ways. Male mice were first studied during repeated pairing with ovariectomized females (41). Both WT and KO mice presented with normal ovariectomized females showed high levels of investigation, demonstrating that they were able to recognize the females and behave accordingly. During repeated pairing with the same female, they showed a characteristic decline in the time spent investigating the female with an almost full recovery following the introduction of a new female (Fig. 8D). ANOVA (genotype by repeated exposure) showed no significant genotype differences [$F(1,40) = 3.02$, $P = 0.09$], a highly significant habituation [$F(1,4) = 50.9$, $P < 0.0001$] and no interaction. Most importantly, both KO and WT showed a parallel significant increase in sniffing after introduction of a new ovariectomized female [WT, $F(20,1) = 24.41$, $P < 0.0001$; KO, $F(20,1) = 24.54$, $P < 0.0001$]. This experiment leads to two important conclusions. The KO mice do not have impairment in female recognition, and they seem to have a normal olfactory behavior that allows recognition of the female as well as allowing them to distinguish between different females.

Olfactory functions were also tested in a olfactory-guided foraging task (42). Both WT and KO mice, tested in several trials, were able to locate chocolate either visible on the litter or buried under the litter at different positions, at the four corners of the cage. The time required to find and get the food was similar for all mice (Fig. 8E). ANOVA (genotype by factorial analysis) showed no significant genotype differences during the first trial [$F(1,50) = 0.82$, $P = 0.37$]. ANOVA (genotype by repeated exposure) showed no significant genotype differences during the successive trials when the chocolate was buried under the bedding [$F(1,50) = 1.11$, $P = 0.298$].

Serum testosterone was also measured, and no differences were found between mutants and WT (0.31 and 0.23 ± 0.05, respectively).

Moderately altered hippocampus synaptic plasticity in mutant mice

Based on the observations of Ishizaki *et al.* (18) that synaptic responses in the hippocampus of *Gdi1* KO mice were altered during moderately high-frequency stimulation, we tested the

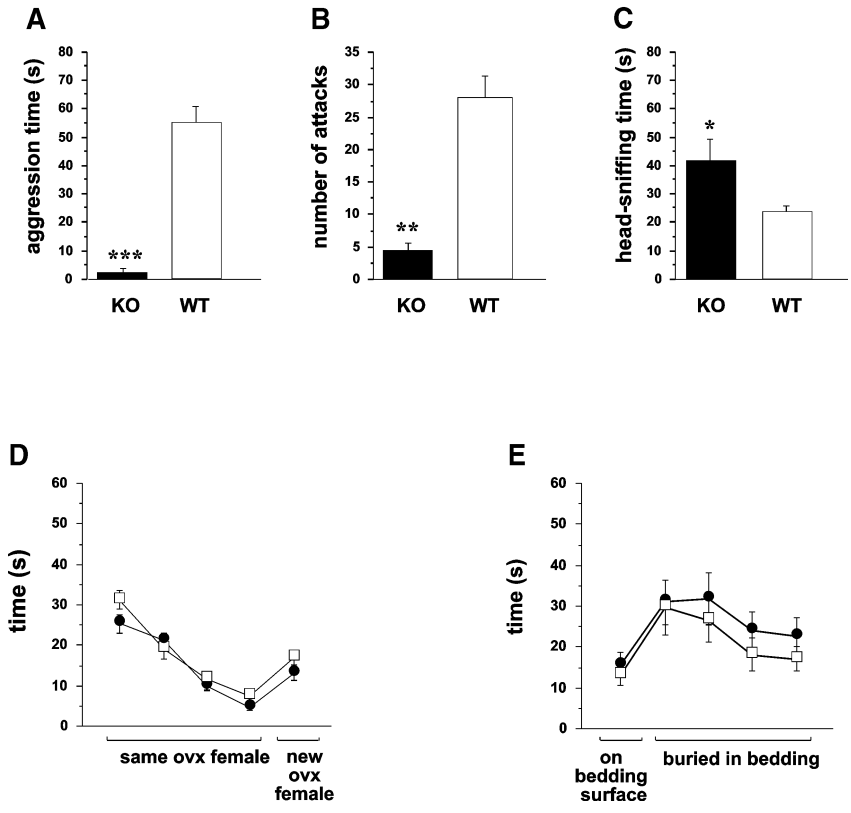

Figure 8. *Gdi1* KO mice display altered male social behavior. (**A**, **B** and **C**) Resident intruder test. Pooled results of the resident-intruder test done on 129B6 mice from the F2 and F3 generations (WT, $n = 19$, white bars; KO, $n = 20$, black bars). Inbred C57Bl/6J mice were used as intruders. The test duration was 600 s. (A) Cumulative duration of the offensive attacks. (B) Total number of attacks. (C) Cumulative duration of the time spent sniffing the snout area of the intruder. The histograms represent the mean and bars the standard error. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$. (**D** and **E**) *Gdi1* KO mice do not show olfaction deficit. Pooled results obtained from 129B6D2 and 129B6 mice from the F2 and F3 generations in two olfactory tests. (D) Repeated pairings with ovariectomized (ovx) females (WT, $n = 21$, open squares; and KO, $n = 21$, closed circles). (E) Olfactory-guided foraging task (WT, $n = 26$, open squares; KO, $n = 26$, closed circles). Data points represent mean ± SE.

hypothesis that long-term plasticity might be affected if it were induced by 5 Hz stimulation. Stimulating for 30 seconds at 5 Hz produced LTP in both genotypes, but the first 5 minutes after tetanus revealed a significant difference between the two groups (Fig. 9). The field EPSP slope was significantly depressed (mean 78.6 ± 4.5%) relative to baseline in KO mice in the first 5 minutes post tetanus ($t = 4.49$, df = 4, $P < 0.02$), while WT mice were not different from baseline (mean 114.5 ± 6.2%, $t = 2.13$, $P > 0.05$) in the first 5 minutes. By 25 minutes after tetanus, both groups are significantly potentiated (WT 133.4 ± 9.8%, $t = 2.7$, $P < 0.05$; KO 112.3 ± 4.3, $t = 2.9$, $P < 0.05$). Consistent with previous observations, we found no differences in LTP following θ-burst tetanus in the CA1 region of the hippocampus (Fig. 9) or in the basolateral amygdala (not shown). θ-Burst tetanus produced no significant differences between the WT and KO mice in the magnitude of LTP at any time after tetanus [2-way repeated measures ANOVA comparing genotype and time in 5-minute bins; $F(1,5) = 0.28$, $P < 0.05$].

DISCUSSION

In this paper, we report the characterization of mice carrying a deletion of the X-linked MR gene *Gdi1* and null for αGdi. *Gdi1* KO mice were viable and fertile and did not present visible morphological or neuropathological alterations. Based on the characterization of their cognitive and behavioral phenotype, we suggest that the *Gdi1*-deficient mice represent a model to study the role of *GDI1* in human MR.

Gdi1 is an ubiquitously and abundantly expressed gene (7), but our results show that it is a dispensable gene in most body tissues and brain regions. Since in mammals there are two *GDI* genes, encoding α- and βGdi, it is likely that βGdi, which is present in normal amounts in the brains of the mutants, can compensate for the lack of αGdi. This seems to be the case for Rab3A, the most abundant Rab GTPase in brain, which was very mildly affected by lack of αGdi. The amounts of total, membrane-bound and soluble Rab3A were almost identical in WT and KO, particularly in synaptosomes, where most Rab3A is localized. Our results explain quite well the electrophysiological phenotype of the *Gdi1* KO mice, which was very different from that of the *Rab3A*-null mice (15,16). Preliminary data from our laboratories showing that the Rab3a KO mice (P. D'Adamo, unpublished observations) do not present cognitive alterations similar to those of the *Gdi1* KO, further confirm this finding. Considering all the data together, we can conclusively say that lack of *Gdi1* does not affect Rab3A, as might have been expected from their relative

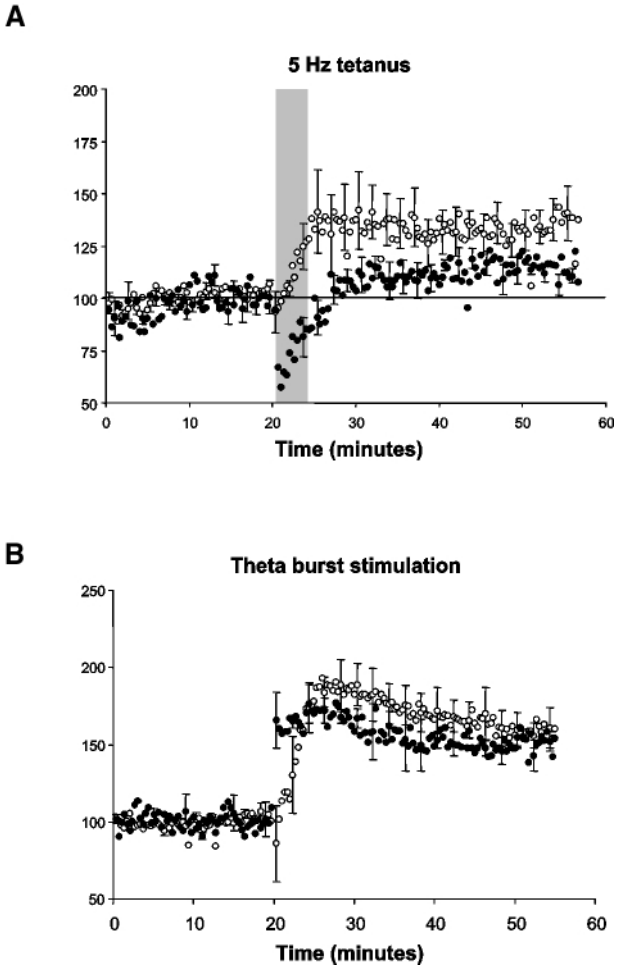

Figure 9. Long-term potentiation in the hippocampus of *Gdi1* KO mice. Collateral/commissural inputs to the CA1 region of hippocampal slices were tetanized using either (**A**) 30 s of 5 Hz stimulation or (**B**) three trains of ten Θ-bursts. Open circles, WT; closed circles, KO.

abundance in brain. Lack of *Gdi1*, however, does affect other Rab proteins that are apparently less efficiently retrieved from membranes by βGdi, and whose altered intracellular distribution may be responsible for the *Gdi1* KO phenotype. The most striking differences were seen in Rab4 and Rab5, which are Rab proteins involved in endocytosis (43,44) and abundant in brain and in the synaptic vesicle fraction (45). Our results suggest that the abnormal distribution and particularly the large amount of membrane-bound Rabs may be the limiting steps for specific endocytic events. Alternatively, the altered composition of the endocytic vesicles and particularly of the synaptic vesicles could be the cause of alterations in vesicle recycling and intracellular trafficking.

The inactivating mutation in *Gdi1* and the consequent alterations in the amount and intracellular distribution of some of the Rab proteins did not cause a generalized malfunction of the mutants brain. We tested the mutant mice in a large set of tasks, and in many we could not see differences between mutant and WT mice. Mutant mice did not present defects in emotional or exploratory behavior. Despite the high level of expression of *Gdi1* in cerebellum, spinal cord and the olfactory bulb, the mice did not have impairments of muscle strength, motor coordination or olfaction. Taste (not shown) and pain sensitivity were tested and appeared normal, indicating that sensory processes were not affected by the mutation.

The *Gdi1* KO selectively impaired hippocampus-dependent tasks important for acquisition of memory across short time intervals. In rodents, the hippocampus has long been recognized as the structure that encodes spatial information, as well as aspects of working and episodic-like memory (28,46). The integrity of hippocampus-dependent reference memory in the *Gdi1* KO was shown by their normal performance in the standard hidden-platform version of the Morris water maze, as well as by their capacity to learn the procedure in the radial maze, a spatial memory-dependent task. Also, hippocampus-dependent one-trial learning, essential for the acquisition of episodic-like memory in the DMP version of the Morris water maze, was unaffected by the mutation.

On the other hand, in the radial maze, working memory, a form of short-term memory that requires the animal to remember trial-dependent information over a relatively short duration, was significantly affected by the *Gdi1* KO. It was suggested that formation of working memory in the eight arm radial maze was a process similar to formation of episodic memory, since in both tasks animals must rapidly establish and maintain memory of visited arms based on single within-trial exposures, and must suppress interference from memory of previous trials (47). Our results show, however, that the two tasks are not identical. We suggest that the impairment in formation of working memory in the *Gdi1* KO is likely due to defects in forming short-term memories.

In agreement with this interpretation are the results obtained in fear conditioning. Trace fear conditioning was drastically altered in the *Gdi1* KO, while the standard version of the fear conditioning test, delayed fear conditioning, done in an identical set-up but not involving time delay, was not affected. Trace fear conditioning tests the capacity to form associations across a short time interval, and may require the formation of short-term memories similar to what is required for working memory formation in the radial maze. Moreover, trace fear conditioning is known to depend on the integrity of the hippocampus, while delay fear conditioning is spared after large hippocampal lesions, and appears to be sensitive to gross amygdala malfunctions (48). In conclusion, we suggest that *Gdi1* is important for hippocampus-dependent molecular mechanism(s) specifically involved in maintaining memory of events across short time intervals and/or in formation of short-term memory.

Altered social behavior is one of the diagnostic parameters of human MR (49). The observation that the *Gdi1* KO mice lacked any aggressive sign towards intruders and showed altered contact behavior was therefore of great interest. This cannot be attributed to reduced levels of testosterone, and olfactory abilities appeared intact. On the other hand, appropriate behavior in critical social interactions such as in the male-intruder paradigm requires an integration of olfactory signals, perception of species-typical display postures in the opponent, and coordination of own responses. This complex interplay appears to depend substantially on the amygdala. In fact, marked alterations in social behavior and reduced inter-male aggression are a hallmark of amygdala lesions, as shown

in monkeys, dogs, rats and golden hamsters (50). Whether the *Gdi1* KO in the amygdala is responsible for the alterations in social behavior or the altered behavior is dependent on the hippocampus-related defects in cognitive functions will have to be determined, and a conditional KO of the *Gdi1* gene is in preparation.

The cognitive and behavioral analysis of the *Gdi1* KO mice revealed a pattern of many spared abilities combined with specific deficits, particularly in the hippocampus. This might reflect localized interaction of αGdi with structure-specific mechanisms, either during development or in the adult brain, as is suggested by the only histological difference detected in the *Gdi1* KO, a lamination defect in the hilar part of the hippocampal CA3 region similar to what is often observed in different WT mouse strains (51,52) and in mutants (53–55). Whether the CA3 alteration plays a functional role is not known, since in no case was it related to a specific phenotype. However, a more parsimonious explanation of our cognitive and behavioral results is that lack of αGdi causes a widespread synaptic impairment that reveals clear deficits only for tasks challenging specific forebrain functions.

During a 30 s train of pulses at 5 Hz, CA3–CA1 synapses in the hippocampus of *Gdi1* KO mice were able to fire, but following the train a pronounced post-tetanic depression resulted, possibly because of depletion of the immediately available vesicle pool. This hypothesis is supported by the observation that θ-patterned stimuli, in which high-frequency stimuli are delivered in short bursts, does not differentiate between *Gdi1* KO mice and controls, either in hippocampus or in amygdala (not shown). This phenotype is consistent with that of other mice with deletions of presynaptic vesicle or vesicle-associated proteins, such as synapsin I and II (56,57), which are also associated with cognitive abnormalities (58) and with the defects in short-term plasticity of the *Gdi1* KO made by Ishizaki *et al.* (18).

The deficit in CA1 synaptic plasticity, specific to one particular induction protocol (that of 5 Hz stimulation), suggests how the same brain structure can contribute to different types of learning and memory, by differential recruitment of synapses by different patterns of activity. Thus, θ burst-like activity might establish the plasticity required to successfully engage spatial reference memory, whereas formation of working memory and trace fear conditioning may depend on activity more similar to relatively prolonged 5 Hz activation. There are significant differences at the level of neurotransmitter receptors and second-messenger systems between long-term potentiation (LTP) induced in CA1 with 5 Hz activity and other patterns (59,60), and this could explain the selectivity of the effects of *Gdi1* KO despite the ubiquitous distribution of αGdi and the generalized decreased amount of many different Rab proteins.

Finally, our findings fit well with human MR, which is characterized by relative sparing of most central nervous system functions. Up to now, no simple correlation of MR with specific neuroanatomical or neurochemical traits has been reported. We believe that the approach of identifying human genes whose mutation is causing MR and to delete them in mice offers some unique perspectives. The availability of a *Gdi1* KO mouse will allow us to search for common physiological deficits in both humans and mice, and the behavioral and physiological analysis of mice will permit a focused search in human patients for deficits underlying their mental handicap. Finally, it may also lead to the discovery of new genes mediating mental faculties and responsible for MR.

MATERIALS AND METHODS

Isolation of the mouse *Gdi1* gene

The *Gdi1* mouse gene was isolated from a phage mouse genomic library made from DNA of 129/Sv mice in λ-DASH II (a generous gift from A. Nagy). The mouse *Gdi1* gene was sequenced starting from the first exon, and the 6752 bp sequence was submitted to GenBank (accession no. AF441240). The murine *Gdi1* gene has the same organization of the human gene (5).

Gene targeting and generation of *Gdi1* KO mice

The targeting vector contained 2.7 kb of the *Gdi1* gene and 5′ flanking region upstream from the ATG and 3.7 kb of DNA downstream from the fourth exon. The promoterless *lacZ* gene and the neomycin-resistance cassette driven by the *PGK1* promoter were cloned in pBSSk–. A 2.7 kb *Bam*HI/*Kpn*I fragment, containing the *Gdi1* promoter and 5′-UTR, and a 3.7 kb *Eco*RV fragment, from exon 4 to exon 11, were subcloned into the vector. After linearization and electroporation into AB1 embryonic stem (ES) cells, G418-resistant colonies were analyzed by PCR, from primers PCR1 (5′-GGTATTGGTTGTTGGGTCCTGAG-3′) and PCR2 (5′-GGCCTTATTCCAAGCGGCTTC-3′) and using the Expand Long Template PCR System (Boehringer). PCR-positive clones were confirmed by Southern blot using 5′ and 3′ external probes. The 5′ flanking probe, S1, was an RT–PCR product of 320 bp from primers 16Am1 (5′-GAGGTCATCGGACAGGTACTGAG-3′) and 16Am2 (5′-GGCAAAGGAGTCATTCCAGAAGG-3′) containing exons 6 and 7 of the 16A 5′ flanking gene. The 3′ probe, S2, was an RT–PCR product from primers EST5 (5′-GAGACAACCTGCAATGACATCAAAG-3′) and EST18 (5′-TCCCAAACCATCCCTAAGGTTAAGG-3′), containing the last 120 bp of exon 11 and 190 bp of the *Gdi1* 3′-UTR. Two homologous recombinant clones were injected into B6D2F1 blastocysts by standard methods. Chimeric mice from both clones were crossed to C57BL/6J or B6D2F1 female mice. Genotypes were determined by PCR from tail DNA using primers G1 (5′-TATAGTAGCATCTTTACCAGCTGAC-3′) and G2 (5′-GAATGCATCCTGTCTGGCATCATG-3′), which amplify a 555 bp fragment of the WT allele, and primers Z1 (5′-GAAGCCGCTTGGAATAAGGCCG-3′) and Z2 (5′-CCCCAGATCAGATCCCATACAATG-3′), which amplify a 443 bp band from the KO allele. Southern and northern blots were done using standard methods.

Animals

All animals were maintained on a 12 h light/darkness cycle at 22–25°C. Food pellets and water were available *ab libitum*,

unless stated otherwise. WT and KO mice were tested at 2–5 months of age.

Histological analysis

Adult mouse brain were dissected, and transversal and coronal 20 µm cryostat sections were hybridized to a DIG-labeled riboprobe (Boehringer). The probe for *in situ* hybridization was the cloned RT–PCR product amplified from primers EST5/EST18 (see above). The corresponding sense probe was used as control. Nissl and Timm stains were performed as previously described (53).

Western blot analysis

Brains from individual mice or pools of two to six animals were dissected, and subcellular fractions were prepared as previously described (19). Proteins in sample buffer were fractionated in 10% SDS–PAGE, and western blots were done using standard methods.

Semiquantitative RT–PCR

Primer pairs were designed for the same Rab proteins analyzed by western blots. RT–PCR was done as previously described (61). The number of PCR cycles was between 25 and 30, and was adjusted for each mRNA to be below saturating levels. The sequences of the primers are available at the website (http://www.sanraffaele.org/research/toniolo/).

Morris water maze

The standard hidden-platform version of the Morris water maze was done as previously described (27).

Delayed matching-to-place (DMP) task

The test was performed as described by Chen *et al.* (29), with a few modifications. Mice were trained in a circular pool of 150 cm diameter using a 14 cm × 14 cm wire-mesh platform. The mice were pretrained to a visible platform, in a different room, for 3 days, for six trials per day, with intertrial intervals of 30 min. During the DMP task, a mouse performed maximally eight trials per day, separated by an inter trial intervals of 10 min. The maximum number of training days was five for platform position 1 and four for platform position 2–5. The animals were trained in the same platform position until they reached the criterion of three subsequent trials with an escape latency $\leq$15 s, or until completing the maximum number of training days for each platform position. If a mouse reached the criterion, the training was stopped. It was started the following day, to a new platform location.

Radial maze

Spatial learning in the eight-arm radial maze was tested as previously described (62). Briefly, food-deprived mice (maintained at 85% of their free-feeding weight) were adapted to the maze for 2 days and then allowed to collect food morsels from a cup at the end of each arm for ten trials per day for 8 days.

Fear conditioning

Apparatus. The conditioning chamber (chamber A: $L \times W \times H$: 25 cm × 17 cm × 23 cm) was an opaque chamber with a grid floor through which scrambled foot shocks could be delivered as US (0.26 mA average intensity). The chamber was placed into a dimly lit (<5 lux) sound-attenuating box (background noise level 55 dB), and a speaker on top of chamber A allowed sound stimuli to be delivered as CS (2000 Hz, 92 dB). Auditory fear conditioning was tested either in chamber A or in chamber B ($L \times W \times H$: 20 cm × 10 cm × 23 cm). Besides being smaller, chamber B had a flat floor covered with scented bedding material. During each trial, freezing as the unconditioned reaction was continuously recorded manually as well as automatically (grid of 24 × 16 evenly spaced infrared beams).

Procedure. All mice were pre-exposed to chamber A for 10 min on the 2 days preceding conditioning to reduce the salience of context cues during conditioning. Animals were either submitted to a delay or trace fear conditioning session. Both sessions consisted of a 1 min adaptation period followed by five identical 3 min conditioning trials. During delay fear conditioning, a trial began with the presentation of the CS (15 s), with the US delivered during the last 2 s of CS presentation. The remaining 165 s formed the intertrial interval. During trace fear conditioning, the only modification was that US delivery followed the end of CS presentation, with a CS–US interval of 15 s. Testing auditory fear conditioning took place in chamber B (24 h after conditioning) or—after 2 days of exposure to reduce reactions to the context—in chamber A (3 and 2 weeks after conditioning). Testing consisted of 1 min without (Bl, baseline) followed by 1 min with the CS turned on.

Resident-intruder test

WT and KO male mice were kept individually for 2 weeks prior to the test, and were used as residents. C57BL/6J or B6D2F1 mice, housed in groups of five, were used as intruders. After transfer of the intruder to the home cage of the resident, the behavior was tape-recorded and the cumulative duration of aggressive behavior (biting attacks and tail rattling) and social behavior (sniffing head or back, and social grooming) were determined.

Social memory

Female recognition was done as previously described (42).

Olfactory-guided foraging task

The olfactory-guided foraging task was done as previously described (42).

Data collection and statistical analysis

Data were collected using the analysis software Wintrack (63). Statistical computations were done using Statview 5.0 (SAS Institute, Cary, NC, USA, (www.statview.com)).

Hippocampal slice recording

Slice preparation and recording were done as previously described (64).

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